Abstract

Immunosuppressive enzyme, indoleamine 2,3-dioxygenase 1 (IDO1) plays a pivotal role in regulating the metabolism of L-tryptophan (L-Trp) through kynurenine pathway, which is a well-established therapeutic target for the treatment of diseases associated with immunosuppression. Under pathophysiological conditions the expression of IDO1 enzyme from its mRNA gets highly up-regulated in response to inflammatory signals (like interferon-γ) within the immune system. Up-regulation of the IDO1 enzyme is directly related with several diseases including cancer, neurodegenerative disorders (Alzheimer's disease), Huntington's disease, schizophrenia, depression, age-related cataract, and HIV-1 encephalitis. Hence, proper understanding of the IDO1 activity will be helpful for the development of novel IDO1 inhibitors as emerging immuno-oncology agents.

On the basis of the experimental works performed during the research period, the content of the thesis entitled **"*Activity Studies of Indoleamine 2,3-Dioxygenase 1 Enzyme*"** have been divided into five chapters. **Chapter 1** of the thesis provides an overview of IDO1 enzyme and demonstrates the activity and regulation of this immunosuppressive enzyme in medicine, life science and other related research fields. These enzymes are responsible for the degradation of an essential amino acid L-Trp, which is required for the protein synthesis and other important metabolic functions. IDO1 mediated depletion of the local L-Trp concentration and accumulation of the kynurenine metabolites regulate the local immunosuppression. High levels of various L-Trp metabolites cause a variety of diseases, such as rheumatoid arthritis, tuberculosis, leukemia, Hodgkins disease, bladder cancer, and prostate disorders. On-going pre-clinical and clinical studies with different cancer models suggest that the IDO1 enzyme assists cancer progression and metastasis. The immunomodulatory role of IDO1 suggests that its inhibition might results in the rejuvenation of the normal immune responses and would enhance the efficacy of cancer treatment. Therefore, key challenges in advancing immunotherapies are the development of specific IDO1 inhibitors and exploration of their mechanism of action.

Chapter 2 describes the optimization of an efficient bacterial expression and purification system for the production of recombinant human IDO1 and TDO enzymes. Purified IDO1 enzyme will greatly assist in screening the synthesized inhibitors. Bacterial

expression conditions and purification system for the production of rhIDO1 and rhTDO enzymes were optimised in our laboratory by varying the incubation temperature, IPTG, ALA concentrations and buffer composition with high concentration of imidazole using Ni^{2+} affinity chromatography, respectively. The purity of the enzyme was assessed by Coomassie-blue stained SDS-PAGE analysis. The UV-visible spectra of IDO1 and TDO enzymes displayed the characteristic Soret and Q band in two different state of iron. The circular dichroism (CD) spectral data in the region of 190-260 nm showed that the secondary structure of rhIDO1 consists of 30.2% alpha helical, 7.9% beta sheet and 64.2% random coil. The thermal denaturation temperature of IDO1 enzyme was found to be 55 ºC. The enzyme activity studies were performed by spectroscopic and HPLC methods. The other enzyme kinetics parameters including K_M, k_{cat}, V_{max} and k_{cat}/K_M were also calculated from spectroscopic measurements. Overall findings suggest that the access of this enzyme assisted in the design of IDO1 inhibitors.

Chapter 3 demonstrates the rational design and synthesis of a series of nitrobenzofurazan derivatives of *N'*-hydroxyamidines and their inhibitory activity against purified IDO1 enzyme under *in vitro* and cellular environments. The optimization leads to the identification of few compounds as potent inhibitors of IDO1 enzyme with IC_{50} value of 39-98 nM. The *in vitro* cellular IDO1 enzyme inhibitory activity of these selected compounds also showed stronger potency (IC_{50} = 50-151 nM) with no/ negligible level of toxicity. The stronger IDO1 enzyme inhibitory properties of these compounds were supported by the molecular modelling studies. The UV-visible spectra showed the direct binding and competitive mode of inhibition of the potent compound with the IDO1 enzyme. The TDO enzyme activity of the potent hydroxyamidine analogues also showed 118 to 1593-fold stronger selectivity for IDO1 enzyme over TDO enzyme. Overall, the experimental results suggest that the compounds with hydroxyamidine and nitrobenzofurazan moieties are potential inhibitors of IDO1 enzyme and can be used as a tool for further development of IDO1-based inhibitors.

To identify a new structural class of IDO1 inhibitors, in the **Chapter 4** a series of pyridopyrimidine, pyrazolopyranopyrimidine derivatives were synthesized and their inhibitory activities were investigated under *in vitro* and cellular environments. Subsequent modification of the electronic properties of the fused heterocyclic ring and substitution of the phenyl ring directed to the identification of potent inhibitors with low micromolar (IC_{50} = 296-473 nM) inhibitory activities against purified IDO1 enzyme. These compounds also showed EC_{50} value in the nanomolar range with no/negligible

cytotoxicity. Spectroscopic studies suggest that the pyrazolopyranopyrimidine derivatives preferably interact with the deoxy-ferrous-IDO1 enzyme. Molecular model structure propose that the electronic properties of pyridopyrimidines and pyrazolopyranopyrimidines ring and halogen substituted phenyl ring assist these compounds to interact with the IDO1 through hydrogen bonding, pi-stacking and hydrophobic interactions. Counter screening against TDO enzyme show the stronger selectivity of the potent compounds (100-300 fold) for IDO1 over TDO enzyme. Overall, these observations suggest that further development of fused heterocyclic compounds could lead to a new class of potent inhibitors of IDO1 enzyme.

IDO1-based drug development has been hindered due to the use of idealized dilute buffer solution media during the screening and optimization of inhibitors, which do not reflect the highly crowded intracellular environment. **Chapter 5** describes the efficacy of external crowding agents in mimicking the cell-like environment for IDO1 activity assay. To mimic the intracellular environment under *in vitro* conditions, chemically inert, non-charged, hydrophilic synthetic polymers polyethylene glycol (PEG) and ficoll were selected as crowding agents. A non-linear relationship between the size and volume of the crowding agent with enzyme kinetics parameters was observed. The results suggest that judicious use of size and volume of the crowding agent could provide cellular mimetic environment. The inhibitory activity study under the optimized crowding conditions showed a similar IDO1 inhibitory potency of the selected potent compounds as in the presence of cellular environment. The circular dichroism (CD) analysis of the IDO1 enzyme showed almost no change in the secondary structure content of the protein. The temperature-dependent CD measurements revealed that the presence of crowding agent increases the thermal perturbation temperature of IDO1 enzyme, indicating the improved stability of the enzyme under the experimental conditions. Overall our findings suggest that IDO1 enzyme activity under *in vitro* crowding environments will be helpful in understanding its enzymatic activity under complex media such as biological fluids.

Our attempt to design small molecules inhibitors for IDO1 enzyme in order to achieve higher selectivity and specificity, led to the development of nitrobenzofurazan derivatives of *N*′-hydroxyamidines and pyridopyrimidine, pyrazolopyranopyrimidine derivatives which showed stronger inhibitory potency for IDO1 enzyme. The development of new IDO1 enzyme activity assay mimicking the intracellular environment was also helpful for designing and screening of the synthesized IDO1 inhibitors. Overall, our findings suggest that structural simplicity, low cytotoxicity,

inactivity for TDO enzyme, similar catalytic efficiency and secondary structural contents of the IDO1 enzyme under *in vitro* crowded conditions helps to understand the therapeutic applicability of the selected crowding agents as well as the inhibitors.